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13. ABSTRACT *(Maximum 200*

This project aims to devise strategies to antagonize the promitogenic action of Ras and thereby suppress the transforming activity of the Erb B2 oncogenes found in 70% of human breast adenocarcinomas. The initial strategy was based on the ability of the Rap-1 GTPase when overexpressed, to suppress the malignant phenotype of V12 Ras transformed fibroblasts. It was anticipated that Rap-1 which shares an identical sequence corresponding to the primary Ras effector binding domain (amino acids (32-44), when overexpressed competes with Ras for critical mitogenic effects. In the past year, we have focused on the identification and characterization of proteins that interact with Rap-1 and Ras through their effector loop, in a GTP dependent fashion. We are attempting to ascertain whether the several proteins we have isolated that exhibit effector loop dependent binding to Rap-1 and Ras actually function as effectors for either Rap-1 or Ras, and whether they participate in regulation of the mitogenic activity of the cell in a positive or negative fashion.

We have initially characterized the protein AF-6 because it was first isolated as a fusion partner of gene ALL-1, a chromosomal translocation found in acute leukemias. AF-6 binds to Ras and Rap-1 in a GTP dependent fashion, with a higher affinity for Rap-1. This contrasts with Raf, which prefers Ras. Future work will examine the ability of AF-6 and other candidate effectors to alter cell growth.

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Joseph Avruch 9/26/96

PI - Signature Date

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Introduction

Breast cancer is believed to arise from a multistep process involving multiple somatic mutations resulting in the generation of oncogenes and loss of suppressor genes. The process is superimposed on an initial genotype that may contain predisposing mutations (e.g. BRCA1) and is influenced strongly by ovarian steroid hormones (1).

The most common dominant oncogenes found in breast cancer arise from activating mutations in the Erb B2 receptor tyrosine kinase (RTK) resulting in a potent continuous promitogenic signal (1, 2). In all cellular systems examined, the mitogenic capacity of RTKs requires the recruitment of the Ras GTPase (3, 4, 5). Ras itself when mutated to a constitutively active (GTPase deficient) form is a very potent oncogene. Such Ras mutations are very unusual in breast cancer (6), but cRas is frequently overexpressed (2), and this phenotype, when it occurs concomitant with Erb B2 activation, a mutation that occurs in 70% of breast cancers, is associated with a particularly poor prognosis (2).

The overall goal of the studies proposed is to better understand the biochemical mechanism by which RTKs, acting through Ras, promote growth in the breast epithelial cell, and to identify strategies that can be used to antagonize the promitogenic signal conveyed by Ras.

Approximately 2 $^1/_2$ years ago, work from this and several other laboratories (reviewed in 7) uncovered the first direct evidence as to the biochemical mechanism by which activated Ras promotes cell growth. We showed that the active, GTP bound form of Ras bound directly to regulatory domain of the protein (Ser/Thr) kinase protooncogene, cRaf-1; in fact all three members of the Raf subfamily (ARaf and BRaf) exhibit this interaction. Moreover, introduction of activated, Ras independent forms of Raf into fibroblasts led to transformation. Earlier work from

our lab had shown that a major substrate of the Raf protein kinase is the dual specificity kinase known as MAPK kinase or MEK (8), which is the immediate activator of the MAP kinase. In fact, several groups showed that expression of constitutively activated forms of MEK was sufficient to give transformation of murine fibroblasts.

These results suggested that the ability of Ras to bind Raf, recruit it to the plasma membrane and initiate its activation provided a sufficient explanation for Ras' potent transforming action, and strategies that interfered with the Ras/Raf interaction were likely to be antimitogenic.

One plausible strategy that provided the initial focus for the present proposal was suggested by the properties of the Ras-related small GTPase, Rap-1. Rap-1 is about 50% identical to Ras in amino acid sequence, and completely identical over the amino acids corresponding to the so-called Ras effector domain, (Ras residues 32-44) (reviewed in 9). Rap-1A was first isolated as a cDNA capable of causing reversion of the morphologic and growth phenotype of V12 Ras transformed fibroblasts (9). The ability of Rap-1 overexpression to revert Ras transformation, together with the identity of the Rap effector domain to that of Ras suggested that Rap-1 might bind Ras' mitogenic effectors, and sequester them in an inactive state. In fact we were able to show that Rap-1 is capable of binding c-Raf in a yeast expression system, in a manner similar to Ras (7). These findings led us to propose that Rap-1, which was known to be expressed in breast, might be recruited to serve as an endogenous Ras antagonist. We therefore proposed to:

1. Create antibody and cDNA reagents necessary for the study of Rap-1 in normal and malignant breast epithelia.
2. Characterize the regulation of the Raf-MAP Kinase pathway in normal and malignant breast epithelia.
3. Examine the effects of Rap-1 overexpression on Ras-directed signal transduction and cell growth.

4. Examine the regulation of Rap-1 activity by extracellular agonists.
5. Determine the nature of the major Rap-1 targets and their relation to Ras action.
6. Examine the control of Rap-1 gene and polypeptide expression in normal and malignant breast tissue.

Body: 8/96-8/96

As described in the 1st years progress report, we had created a satisfactory polyclonal anti Rap-1 anti serum (Task1) and initiated studies on the regulation of Rap-1 *in situ* and its interaction with Raf-1 (Tasks 2, 4). In addition, we had initiated an expression cloning effort to isolate the major Rap-1-associated cellular proteins that might account for Rap-1's anti Ras action, and other of Rap-1's cellular effects. This latter effort (Task5) proved to be unusually productive, and led to the isolation of several sets of novel candidate Rap-1 and Ras interacting proteins. Two of these sets comprised polypeptides whose sequence encoded motifs that gave clear indication of their catalytic function. One set, of course, were the Raf family kinases, whose binding to Rap-1 had been observed by us, and whose role in Ras signaling is well established. A second set of Rap-1 interactors consisted of four distinct polypeptides which each encoded a domain homologous to the catalytic domain of guanyl nucleotide exchange proteins for small GTPases of the Ras superfamily. These four proteins included:

1. cDNAs identical to those previously reported as encoding a Ral specific exchanger (Ral GDS) (10-13).
2. cDNAs sharing about 60% identity to Ral GDS, reported by several groups as Ral GDS-like (RGL) (11).
3. cDNA encoding a protein about 30% identical to Ral GDS and RGL, which was recently reported as RLF (14).
4. One novel, as yet unreported polypeptide which contains a GDS catalytic domain approximately 30% identical to several functionally

characterized GDS enzymes, including the Ras specific in m-SOS, the Rap-1 specific C3G, etc.

In addition to these two categories of catalytic polypeptides, we recovered 5 other categories of cDNA encoding polypeptides which lacked catalytic domains, although several encoded protein domains known to be important in protein-protein or protein-lipid interaction, such as a zinc finger domain, a pleckstrin homology domain, ankyrin (ANK) repeats, etc.

Each of these polypeptides, like Raf, interacted both with Rap-1 and Ras in a yeast expression system, in an effector domain-dependent way, indicating that the bound preferentially to the GTP charged forms of the GTPases, and like Raf, were candidate effector molecules. At this point we were faced with the choice of devoting effort primarily to the further characterization of these new candidates (Task5) or proceeding with the descriptive studies of Rap-1 overexpression and regulation (Tasks 2 and 3).

Our decision was strongly influenced by emerging reports from a number of laboratories which indicated that multiple Ras-activated pathways in addition to Raf appeared be required for the transforming action of Ras in many cell backgrounds, (15, 16). Moreover, activated Raf was not capable of transforming a variety of epithelial cell lines that were potently transformed by V12 Ras, including the MCF-10A human breast epithelial cell line (17). This result indicated that although Raf remained an important effector of Ras, other mitogenic effectors, remained to be identified, including elements that were especially important in breast and other epithelial lines (e.g. colonic epithelia, etc.). We therefore elected to focus our effort on the characterization of the novel candidate Ras and Rap-1 effectors we had cloned during the initial period.

Our general strategy in approaching this characterization has been to:

1. Express the Rap/Ras binding domain of the candidate and establish that the protein segment is capable of a direct, GTP dependent interaction with the GTPase.
2. Compare the relative affinity of the candidate for Rap-1 GTP versus Ras GTP.
3. Isolate full length cDNA corresponding to the candidate effector.
4. In studies yet to be carried out, we will examine the effect of overexpression of the candidate, both as a wildtype protein, and modified by addition of a membrane localization signal (using the Ki Ras carboxyterminal prenylation motif), both in fibroblast and in the MCF 10A breast epithelial cells. We will examine the ability of the candidate effectors to alter cell proliferation in comparison to the effects of active mutants of Ras and Rap-1. This will greatly expand the work originally described as Task3, but will provide much more significant information as regards the identity of the important mitogenic regulators in breast epithelia.

Several other laboratories have reported initial studies on the interaction of Ral GDS, RGL and RLF with Ras and Rap 1 (14, 18), and we have not pursued these candidates. Interestingly, Herrmann, et al. (18) reported that whereas Ras binds Raf much more tightly than does Rap-1, by contrast, Ral GDS shows a much greater affinity for Rap-1 than for Ras. The effects of Ral GDS on mitogenic responses however are not yet known.

We have chosen to characterize the candidates that have not been examined elsewhere. The candidate we have examined first is a protein called AF-6.

AF-6 was originally cloned (19) as one of the multiple 3' fusion partners of ALL-1, a gene involved in the chromosome 11q 23 translocations present in 5% of acute myeloid and lymphoblastic leukemia, and in the majority of infant

GTP Ras 61L was mixed with various amounts of GTP-γ-S-Rap-1b or GTP-γS-Ras proteins and a fixed amount of GST, GST-Raf (1-257) or GST-AF-6 (28-228) was then added. The GST fusion proteins (and the associated proteins) were recovered by addition of glutathione sepharose beads. The ^{32}P-γGTP radioactivity remaining bound to the washed beads was determined and, after subtraction of the ^{32}P-γ-GTP bound to GST alone, plotted against the concentration of the unlabeled competitor Rap-1 or Ras protein added (Fig. 3). GTP-γ–S-Ras-displaces ^{32}PγGTP- Ras from GST-Raf (1-257) more effectively than does GTP-γ–S-Rap-1b (IC_{50} 20 μM vs 47 μM); thus GTP-γ–S-H-Ras has a higher affinity for Raf (1-257) than does GTP-γ–S-Rap-1b, as previously reported. By contrast, the binding of ^{32}PγGTP- Ras to GST-AF-6 (28-228) is displaced more effectively by GTP-γ–S-Rap-1b than by GTP-γ–S-H-Ras, (IC_{50} 8.4 μM vs 15.2 μM), indicating that GTP-γ–S-Rap1b has a higher affinity toward AF-6(28-228) than does GTP-γ–S-Ras.

Thus the aminoterminal region of AF-6 demonstrates a specific, direct interaction with both Rap-1b and Ras. The interaction requires an intact effector domain and is GTP dependent. The AF-6 aminoterminus binds more avidly to Rap-1b-GTP than to Ras-GTP, whereas the cRaf-1 aminoterminus exhibits a higher affinity for Ras-GTP than for Rap-1-GTP. Herrmann, et al. (18), used the ability of the Raf aminoterminus to inhibit the dissociation of guanyl nucleotides from Ras and Rap-1, to measure the affinity of Raf (51-131) for the two small GTPases. They observed that Raf has a 50-100 fold higher affinity for Ras-GTP than for Rap-1-GTP, whereas the Ras binding segment (the 127 carboxyterminal amino acids) of the Ral guanine nucleotide exchange factor (Ral-GEF), another putative Ras target, exhibits a 100 fold higher affinity for Rap-1 over Ras in the same assay. The present results are in agreement as to the preference of Raf for Ras over Rap-1, although we observe a much smaller difference in relative affinity; it is likely that this quantitative

discrepancy arises from the different assay methods employed. Nevertheless, we conclude that although Raf and AF-6 can each bind to Ras and Rap-1, the higher affinity of AF-6 (and, as previously reported, Ral-GEF reference 18) for Rap-1 over Ras suggests strongly that AF-6 and Ral-GEF are regulated primarily by Rap-1 *in situ*, whereas Raf-1 is primarily a Ras effector.

AF-6, in addition to the Rap-1/Ras binding domain near its aminoterminus, contains several other recognizable domains including a DHR/GLGF (also called PDZ) domain (22), which is found in such proteins as the Drosophila tumor suppressor Dlg-R, human neuronal nitric oxide synthase, the protein tyrosine phosphatases PTP-BAS, PTP-H1, and PTP-MEG, the protein (Ser/Thr) kinases LIMK and MAST-205, the guanine exchange factor Tiam-1 and others. The PDZ domain is thought to directly mediate the formation of macromolecular signaling complexes, and the crystal structure of a PDZ domain has been solved. Many PDZ domain-containing proteins are localized to sites of cell-cell contact, such as postsynaptic, septate and tight junctions. If AF-6 is also localized to such sites, it would imply that Rap-1 is involved in the organization of, or signalling from sites of cell-cell contact. AF-6 also contains a domain homologous to a region in the carboxyterminal tail of the kinesin motor protein, unc-104, and another region homologous with the DIL domains found in the carboxyterminal tails of several class V myosins (Fig. 1). The functional significance of the unc-104 and DIL domains is unknown (23). AF-6 is 40% identical in amino acid sequence to the Drosophila protein Canoe (over 1545 amino acids), and the two proteins exhibit a conserved domain structure (24). Whereas a null mutation in Canoe is lethal, a P element insertion((Cno^{mis+}) gives a "rough eye" phenotype, and shows additional phenotypes in bristle and wing development similar to those seen with mutations in the Notch signalling pathway. Dual mutations in Canoe and Notch related genes, such as Notch itself, shaggy and scabrous suggest significant genetic interactions, and therefore a role for Canoe, and perhaps AF-6, in the Notch pathway.

Conclusion:

The overall goal of this work remains unchanged, namely to identify strategies to interfere with Ras directed mitogenesis, which can be used to interfere with the promitogenic action of the activated Erb B2 oncogene. The specific tasks however, have evolved from the initial proposal, because of the significant evidence for the crucial participation of Ras effectors other than Raf in the mitogenic action of Ras in breast epithelia. We intend to systematically characterize the biologic response to these newer candidates Ras and Rap-1 effectors, so as to ascertain whether.

1. They are expressed in normal and malignant breast epithelia.
2. The impact of their overexpression, singly and in combination on the growth properties of breast epithelial and fibroblast cell lines, in comparison to Ras and Rap-1.

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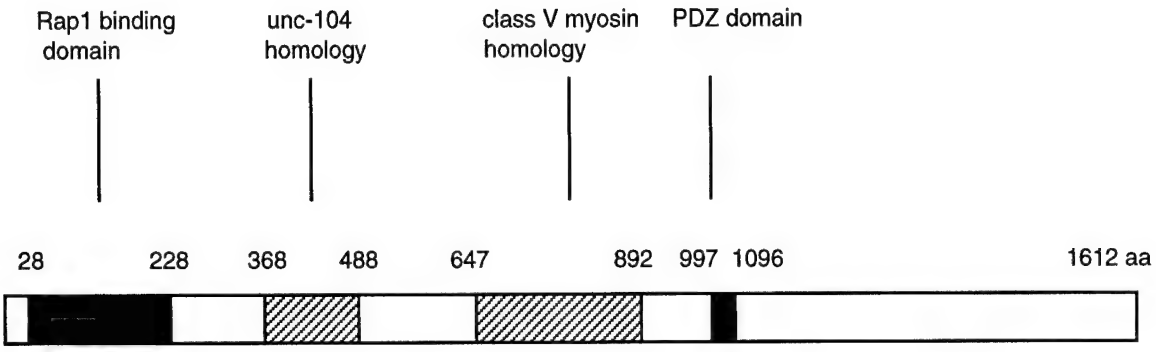
Table I Two-hybrid interactions of AF-6(28-228) with different proteins

	pACT-				
	c-Rap1b	v-H-ras	v-H-Ras[A38]	RalA	c-Raf-1
pAS-AF-6(28-228)	blue	bllue	white	white	white



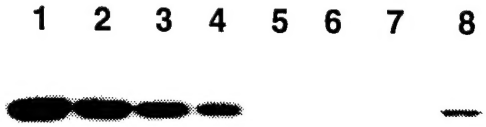
Human AF-6

Fig. 1 **localization if the Rap1 binding domain in human AF-6**

Figure 2a. Direct interaction of AF-6 with Rap1b

	A	B	C	D	E	F	G	H	I	J	K	L
µi Rap1b-GTP-γ-S	20	20	10	5	2	0						
µl Rap1b-GDP-β-S							20	10	5	2	0	20
µl GST-AF-6(28-228)		20	20	20	20	20	20	20	20	20	20	
µl GST	20											20

Figure 2b. Direct interaction of Af-6 with H-Ras



	1	2	3	4	5	6	7	8	9	10	11	12
µl H-Ras-GTP-γ-S	20	10	5	2	0	20						
µl H-Ras-GDP-β-S							20	20	10	5	2	0
µl GST-AF-6(28-228)	20	20	20	20	20			20	20	20	20	20
µl GST						20	20					

Figure 3a. **Comparison of the relative affinities of AF-6(28-228) for Rap1b and H-ras**

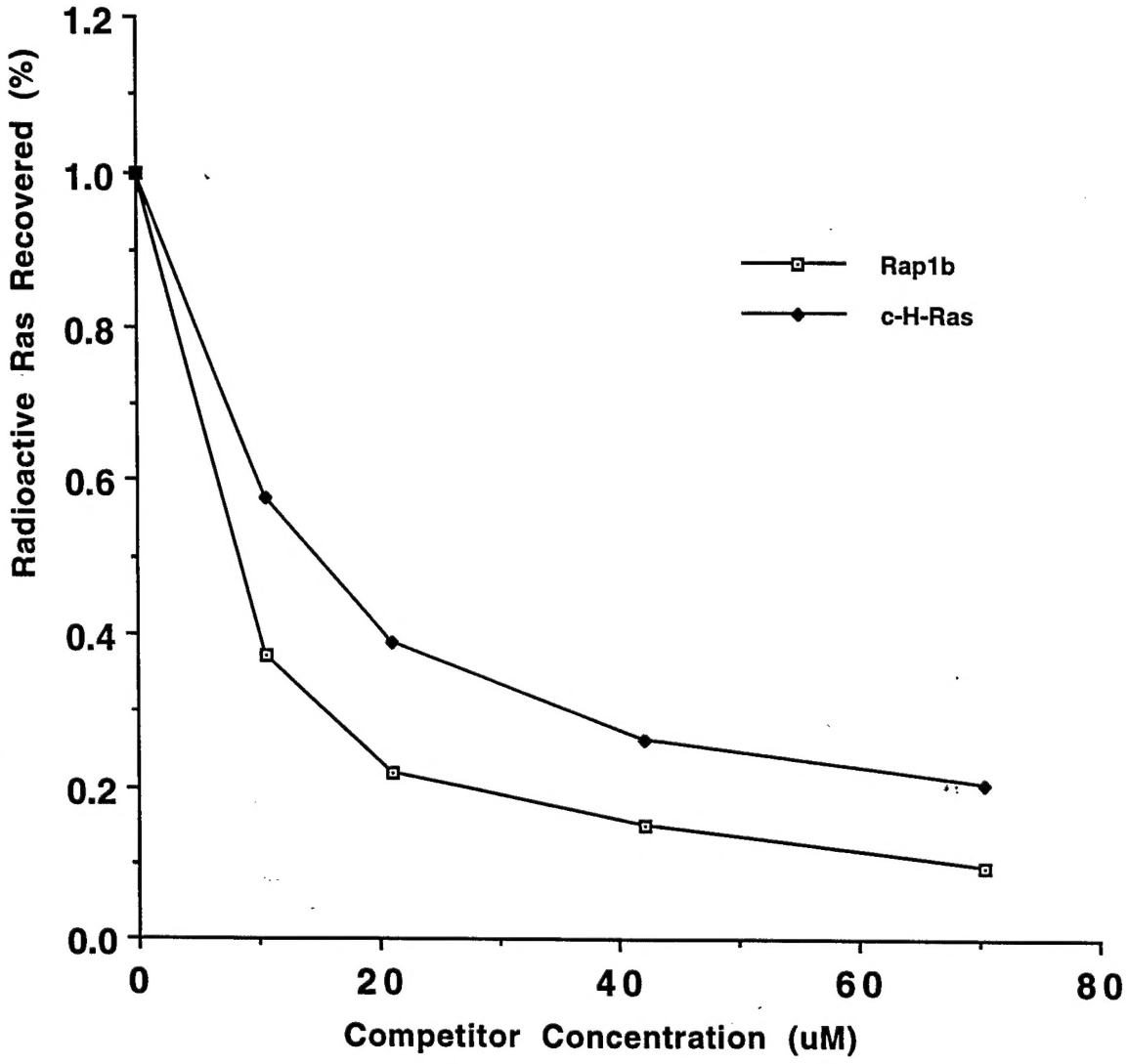


Figure 3b.

Comparison of the relative affinities of Raf(1-257) for for Rap1b and H-r